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ISOENZYME ELECTROPHORESIS OF 30 ISOLATES OF *GIARDIA* FROM HUMANS AND FELINES

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Abstract. Thirty isolates of *Giardia duodenalis* from humans and felines were compared by isoenzyme electrophoresis. Using 10 enzyme systems, 13 different zymodemes were distinguished. The majority of zymodemes could be divided into two groups: one group comprising human and feline isolates with worldwide geographic distribution; the other group containing human isolates restricted to Western Australia. A number of isolates showed multiple-banded patterns and the genetic significance of these findings is discussed. The marked heterogeneity of *G. duodenalis* demonstrated in this study is considered in relation to the epidemiology of giardiasis. The findings are consistent with felines serving as a reservoir of infection to humans.

Giardia duodenalis (syn. *G. intestinalis*; *G. lamblia*) is a flagellated protozoan parasite found in the small intestine of a variety of animals including humans. It occurs throughout the world and is the most frequently reported intestinal parasite of humans in the United Kingdom, United States, and Australia.[1–3] Infection is transmitted predominantly via a fecal-oral route and thus disease occurrence is related to sanitation and hygiene levels, although water-borne and sexual transmission also take place.[2, 4] Infection with *Giardia* may be asymptomatic or characterized by diarrhea, flatulence, abdominal pain, fatigue, and anorexia. In some cases, chronic infections develop, resulting in severe malabsorption syndromes.[5–7]

The epidemiology of giardiasis has been complicated by taxonomic confusion. Early workers classified largely on the basis of host occurrence and more than 40 species were recognized.[8–10] Filice[10] criticized previous cross-transmission studies and recognized only 3 species on morphological grounds: *G. agilis*, *G. muris*, and *G. duodenalis*. Since then most controversy has been associated with isolates assigned to the species *G. duodenalis*. These appear morphologically identical but occur in a wide range of mammalian host species and often exhibit differences in behavioral characteristics such as virulence, infectivity, antigenicity, and susceptibility to drugs.[5, 11–16] Of particular interest is the extent of interspecies transmission, especially between humans and other animals. Conflicting evidence from different laboratories and poor experimental design (reviewed in Woo[17]), mean that cross-transmission studies provide little or no evidence on the zoonotic potential of *Giardia* in lower animals. What is needed is a measure of the genetic differences between isolates of *Giardia* from the same and different host species. Filice[10] and Woo[17] recognized the limitations of morphology and host specificity for this task and concluded that more refined methods are required. Such methodology is now available in the form of isoenzyme and DNA analyses, both of which have been shown to be of great value in determining the extent of inter- and intraspecific variation in protozoan, helminth, and arthropod parasites.[12] To date, the limited application of these procedures has not only confirmed the heterogeneity of *G. duodenalis* but also helped to elucidate the epidemiology of giardiasis.[18–21]

In this study, we report the results of a comparison of 30 isolates of *G. duodenalis* from humans and lower animals by isoenzyme electrophoresis using 10 enzyme systems.

MATERIALS AND METHODS

Giardia duodenalis *isolates*

We have used the term isolate for each sample of *Giardia* received by our laboratory. The isolates of *Giardia* examined in this study are shown in Table 1. Twenty-two isolates from humans

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TABLE 1
Isolates of G. duodenalis

Code	Zymodeme	Host	Geographic origin*	Source†	Nature of initial sample
BAH1	M4	Human	Wyndham, WA	SHL	Cysts
BAH2	M4	Human	Woodanilling, WA	SHL	Cysts
BAH3	M1	Human	Lockridge, WA	SHL	Cysts
BAH4	M1	Human	Derby, WA	SHL	Cysts
BAH5	M1	Human	Marble Bar, WA	SHL	Cysts
BAH6	M1	Human	Warburton, WA	SHL	Cysts
BAH7	M7	Human	Katanning, WA	SHL	Cysts
BAH8	M1	Human	Wickham, WA	SHL	Cysts
BAH9	M1	Human	Marble Bar, WA	SHL	Cysts
BAH10	M1	Human	South Hedland, WA	SHL	Cysts
BAH11	M1	Human	Kalgoorlie, WA	SHL	Cysts
BAH12	M8	Human	Wyndham, WA	SHL	Cysts
BAH13	M1	Human	Kelmscott, WA	SHL	Cysts
BAH14	M3	Human	Kununurra, WA	SHL	Cysts
BAH15	M12	Human	Kununurra, WA	SHL	Cysts
BAH16	M9	Human	Derby, WA	SHL	Cysts
BAH17	M1	Human	Adelaide, SA	UA	Cysts
BAH18	M3	Human	Byford, WA	SHL	Cysts
BAH19	M11	Human	Kununurra, WA	SHL	Cysts
BAH20	M10	Human	Kununurra, WA	SHL	Cysts
BAH21	M2	Human	Busselton, WA	SHL	Cysts
BAH22	M1	Human	Carnarvon, WA	SHL	Cysts
BAC1	M6	Cat	Shenton Park, WA	CR	Trophozoites
BAC2	M5	Cat	Murdoch, WA	MU	Trophozoites
BAC3	M4	Cat	Murdoch, WA	MU	Cysts
106	M4	Human	Brisbane, QLD	QIMR	Trophozoites
120	M4	Human	Brisbane, QLD	QIMR	Trophozoites
141	M4	Human	Port Moresby, PNG	QIMR	Trophozoites
P1	M4	Cat	Portland, USA	QIMR	Trophozoites
BAR1	M13	Rat	Murdoch, WA	MU	Trophozoites

* WA = Western Australia, SA = South Australia, QLD = Queensland, PNG = Papua New Guinea, USA = United States.
† SHL = State Health Laboratories of WA, CR = Cat refuge, MU = Murdoch University, QIMR = Queensland Institute of Medical Research, UA = University of Adelaide.

(BAH1-BAH22) and 3 from domestic cats (BAC1-BAC3) were established as axenic cultures in our laboratory from cysts or trophozoites. BAH17 is a multidrug-resistant isolate from a patient who failed to respond to chemotherapy. Three human (BRIS/83/HEPU/120, 106, and 141) and 1 cat (P1) isolates, were provided by P. Boreham, Queensland Institute of Medical Research; 120 and 106 are thought to be clones derived from parent strains using limiting dilution in semi-solid agar. The Portland 1 isolate (P1) was acquired from the American Type Culture Collection (ATCC). This isolate, identified as ATCC No. 30888, was originally believed to be of human origin in 1976, but shows characteristics of the cat-1/Portland isolate.[18, 19] A rodent isolate (BAR1) was originally obtained by the authors from a naturally infected rat and subsequently passaged through laboratory mice. Unlike all other isolates used in this study, BAR1 has repeatedly failed to establish in vitro. All isolates utilized in the present investigation conformed to the *G. duodenalis* morphological group described by Filice.[10]

Collection of trophozoites

Trophozoites for in vitro culture were initially obtained from the small intestine of infected animals or by separating cysts from fecal specimens and incubating them in a low pH excystation medium.[11] *Giardia* isolated from a rat failed to establish in vitro, and thus infections with this isolate were established in 12-day-old outbred Quackenbush/Swiss mice. Trophozoites were harvested from mice by slitting open the small intestine longitudinally. The intestine was then placed in 15 ml of cold (4°C) attachment medium (3 g Tris-HCl, 0.8 g $MgCl_2$, 1 g NaCl, 0.45 g KCl, 0.5 g L-cysteine-HCl, 0.5 g ascorbic acid, 500 ml distilled water, pH 7.0) and shaken for 1 min. The intestine was removed and the suspension

TABLE 2
Enzymes assayed and buffer systems used in electrophoretic analysis of G. duodenalis

Enzyme	Abbreviation	EC* number	Buffer system†
Esterase	EST	EC 3.1.1.1	2
Glucose-6-phosphate dehydrogenase	G6PD	EC 1.1.1.49	1
Glutamate-oxaloacetate transaminase	GOT	EC 2.6.1.1	2
Glucose phosphate isomerase	GPI	EC 5.3.1.9	4
Hexokinase	HK	EC 2.7.1.1	3
Malate dehydrogenase	MDH	EC 1.1.1.37	1
Malic enzyme	ME	EC 1.1.1.40	2
Nucleoside phosphorylase	NP	EC 2.4.2.1	1
Phosphoglucomutase	PGM	EC 2.7.5.1	3
6-Phosphogluconate dehydrogenase	6PGD	EC 1.1.1.44	1

* Enzyme Commission.
† See text for code.

poured into 15 ml plastic tissue culture tubes. The culture tubes were incubated vertically in a 37°C water bath for 30 min. Sedimented debris was then removed from the bottom of the tube and both attached and free swimming trophozoites collected after two washes in cold (4°C) phosphate buffered saline (PBS).

In vitro cultivation

Isolates were originally established axenically in modified bile supplemented with BI-S-33.[11] When subculturing each isolate, flasks were inoculated from the original culture, which was maintained and used for subsequent subcultures. Trophozoites were subcultured in 150 ml glass milk dilution bottles into which 4 glass test tubes (10 × 1.5 cm) had been inserted to increase surface area. Trophozoites were harvested in late log phase by chilling flasks in an ice bath for 30 min, followed by two washes in cold (4°C) PBS. Packed trophozoites from subcultures were immediately frozen and stored at −70°C.

Electrophoresis

Lysates were prepared by thawing frozen subcultures and homogenizing with an equal volume of stabilizing solution (10 g sucrose, 0.1 ml mercaptoethanol, 20 mg bromphenol blue, 100 ml distilled water). Lysates could be refrozen and thawed twice without significant loss of enzyme activity.

Lysates were absorbed onto 3 × 5 mm tabs of filter paper (Whatman No. 3), inserted along a transverse cut in a 12% starch gel (0.67 Sigma hydrolyzed starch: 0.33 BDH soluble starch) and electrophoresed for 4–12 hr at 4°C. Four different buffer systems were employed: 1) Tris-citrate, pH 8.0;[22] 2) Tris-borate EDTA, pH 8.6;[23] 3) Tris-maleate, pH 7.4;[24] and 4) sodium-phosphate, pH 7.0.[25]

Following electrophoresis, gels were sliced and stained for various enzyme systems using methods modified from Harris and Hopkinson.[26] Interpretable and reproducible results were obtained for 10 enzymes (Table 2). No activity was detected for the following enzymes: adenosine deaminase, adenylate kinase, alcohol dehydrogenase, alkaline phosphatase, arginine phosphokinase, catalase, glutamate dehydrogenase, α-glycerophosphate dehydrogenase, isocitrate dehydrogenase, L-leucyl-glycyl-glycine-peptidase, lactate dehydrogenase, mannose-6-phosphate isomerase, sorbitol dehydrogenase, superoxidase dismutase. Activity was detected for acid phosphatase, L-leucyl-proline-peptidase, and L-leucyl-L-tyrosine-peptidase, but band resolution was poor and the results are not reported.

At least two subcultures of each isolate were used for every enzyme. Occasionally, samples of culture media were electrophoresed to check for the presence of contaminating enzymes, but none were detected.

Terminology

In line with accepted terminology for parasitic protozoa,[27] the arrangement of one or more bands displayed by an enzyme is referred to as a pattern and the series of patterns produced by a given isolate for the 10 enzymes scored is called an enzyme profile. A zymodeme is a group of one or more isolates with the same enzyme profile. The term isoenzyme is used to refer to the different molecular forms of an enzyme which pro-

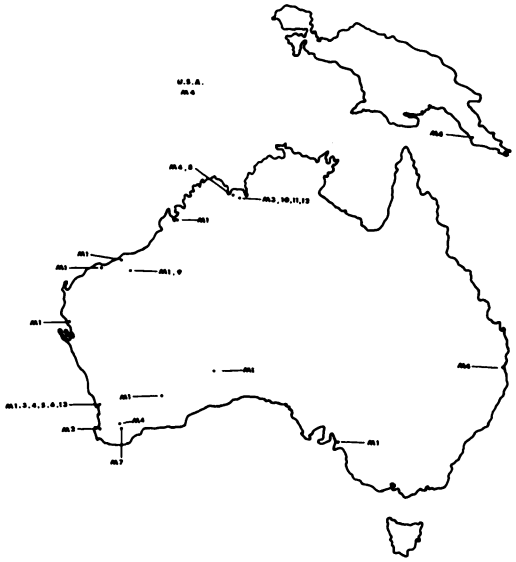

FIGURE 1. Geographic distribution of zymodemes of *G. duodenalis*.

duce bands with different mobility, regardless of whether they are determined genetically or by post-translational modification.

Analysis of electrophoretic data

As we could not confidently assign genotypes to all enzyme patterns, the method we chose to calculate similarities between zymodemes treated isoenzyme bands as phenotypic characters. Each isoenzyme was scored as present or absent for every zymodeme. Euclidean distance was used as a measure of dissimilarity between zymodemes.[28] This ranges from 0, for identical enzyme profiles, to 1, for zymodemes with no shared presence or absence of isoenzymes. Three clustering strategies (single linkage, complete linkage, and group-average or UPGMA[29]) were applied to the dissimilarity matrix using the TAXAN 2 package.[30]

RESULTS

With 10 enzymes, 13 zymodemes were found amongst the 30 isolates examined (Table 1). Zymodeme M1 contained 10 isolates from humans in Western Australia (BAH3, BAH4, BAH5, BAH6, BAH8, BAH9, BAH10, BAH11, BAH13, BAH22) and 1 from South Australia (BAH17). Zymodeme M2 contained a single human isolate from Western Australia (BAH21) and zymodeme M3 contained 2 isolates (BAH14, BAH18), also from humans in Western Australia. Zymodeme M4 contained 2 isolates from humans in Western Australia (BAH1, BAH2), 2 isolates from humans in Queensland (106, 120), 1 isolate from a human in Papua New Guinea (141), 1 isolate from a Western Australian cat (BAC3), and the Portland isolate (P1). Zymodemes M5 and M6 contained single isolates from Western Australian cats (BAC2, BAC1, respectively). Zymodemes M7 to M12 contained single isolates from humans in Western Australia (BAH7, BAH12, BAH16, BAH20, BAH19, BAH15, respectively). Zymodeme M13 contained an isolate from a rat in Western Australia (BAR1). The geographic distribution of the 13 zymodemes is shown in Figure 1.

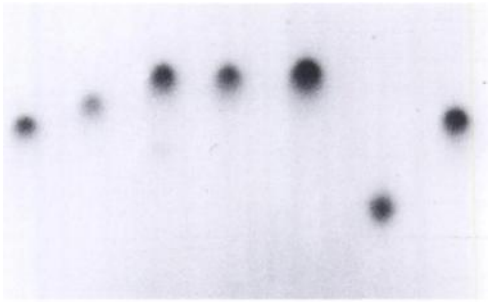

FIGURE 2. Starch gel showing hexokinase staining patterns for zymodemes M4, M11, M8, M12, M10, M13, and M4 (left to right).

Every enzyme showed differences between 3 or more zymodemes. Representative patterns for hexokinase and phosphoglucomutase are shown in Figures 2 and 3. The enzyme profile of each

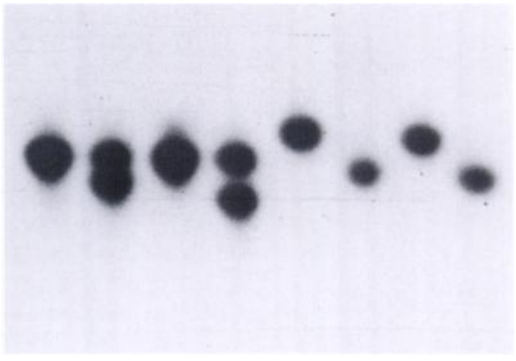

FIGURE 3. Starch gel showing phosphoglucomutase staining patterns for zymodemes M12, M9, M11, M10, M1, M4, M2, and M4 (left to right).

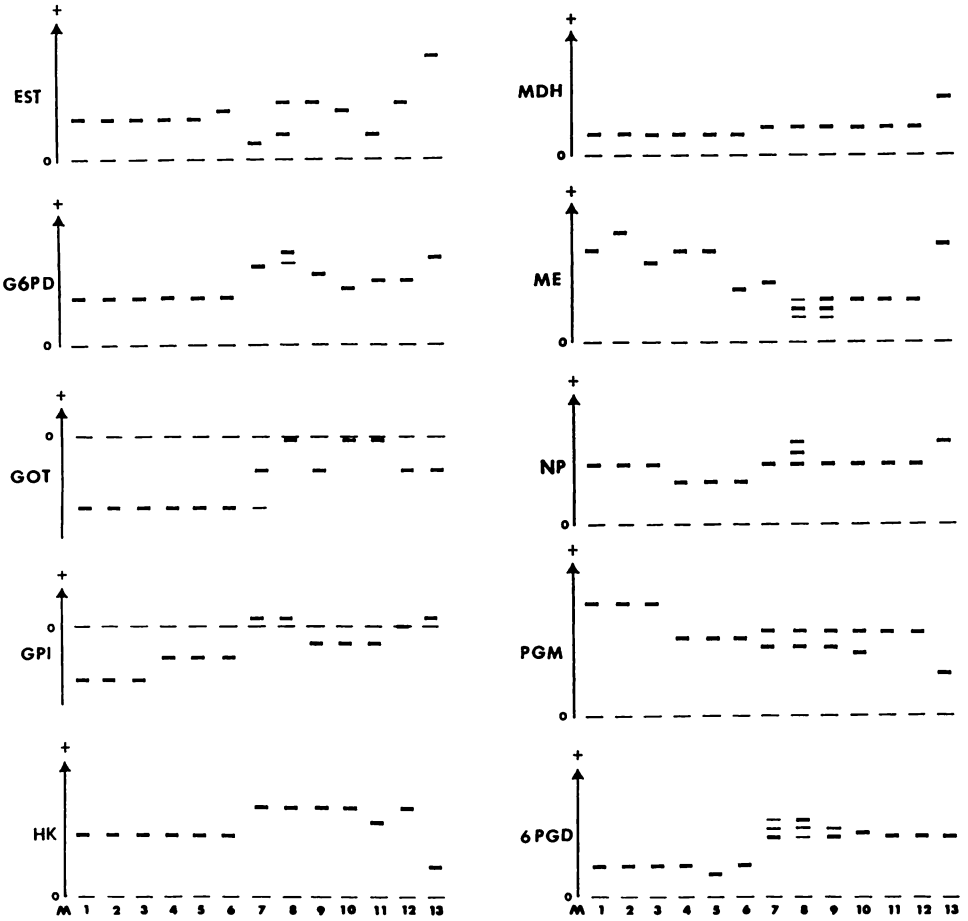

FIGURE 4. Diagrammatic representation of the enzyme profiles of the 13 zymodemes obtained for *G. duodenalis* using 10 enzyme systems. See Table 2 for details of enzymes. Thickness of bands indicates relative staining intensity. Thin dashed line at 0 indicates point of insertion of samples.

zymodeme is shown in Figure 4. Nine zymodemes contained single-banded patterns for all enzymes. Four zymodemes, each containing 1 isolate, had multiple-banded patterns; M7 for 3 enzymes (GOT, PGM, 6PGD), M8 for 6 enzymes (EST, G6PD, ME, NP, PGM, 6PGD), M9 for 3 enzymes (ME, PGM, 6PGD), and M10 for 1 enzyme (PGM). The relative staining intensity of each isoenzyme in the multiple-banded patterns, estimated visually, is indicated in Figure 4. There were no differences in any enzyme pattern between subcultures of the same isolate. For 5 subcultures of BAH7 (M7), 5 subcultures of BAH12 (M8), 3 subcultures of BAH16 (M9), and 3 subcultures of BAH20 (M10), relative staining intensities of isoenzymes in multiple-banded patterns were identical.

Differences in enzyme profiles between zymodemes, measured by euclidean distance, ranged from 0.037 to 0.537 (Table 3). These values are likely to underestimate true phenetic differences beween zymodemes because the method of calculating euclidean distance gave equal weight to shared absence and shared presence of isoenzymes. Comparable phenograms were produced by all clustering methods; that constructed by the group-average strategy is shown in Figure 5. There were 3 principal clusters, the first (group I) incorporating zymodemes M1 to M6, the second (group II) zymodemes M7 to M12, and the third (group III) zymodeme M13. Marked substructuring occurred only in group I, with zymodemes M1, M2, and M3 clustering separately from M4, M5, and M6.

TABLE 3
Matrix of euclidean distances between zymodemes of G. duodenalis

M2	0.037											
M3	0.037	0.037										
M4	0.111	0.148	0.148									
M5	0.148	0.185	0.185	0.037								
M6	0.185	0.185	0.185	0.074	0.111							
M7	0.370	0.370	0.370	0.407	0.407	0.407						
M8	0.500	0.500	0.500	0.537	0.537	0.537	0.278					
M9	0.407	0.407	0.407	0.444	0.444	0.444	0.222	0.204				
M10	0.352	0.352	0.352	0.389	0.389	0.352	0.315	0.333	0.241			
M11	0.333	0.333	0.333	0.370	0.370	0.370	0.296	0.278	0.222	0.167		
M12	0.333	0.333	0.333	0.370	0.370	0.370	0.222	0.278	0.148	0.204	0.148	
M13	0.370	0.370	0.370	0.370	0.370	0.370	0.333	0.426	0.370	0.389	0.333	0.296
	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12

DISCUSSION

Enzyme electrophoresis has recently been applied to answer fundamental questions about the genetics and ploidy of parasitic protozoa.[31, 32] This involves a genetic interpretation of the banding patterns exhibited by isolates. Previous studies of *Giardia* have shown predominantly single-banded patterns, with different isolates often having different isoenzymes.[18, 20, 21, 33] This suggests that *Giardia* is an asexual, haploid organism, with variant enzymes the result of occasional mutations. Most isolates of *Giardia* which were used in the present study also fit this interpretation. However, isolate BAH12 showed multiple-banded patterns for 60% of the enzymes examined, BAH7 and BAH16 for 30%, and BAH20 for 10%. It is unlikely that these multiple-banded patterns resulted from post-translational changes in one primary gene product.

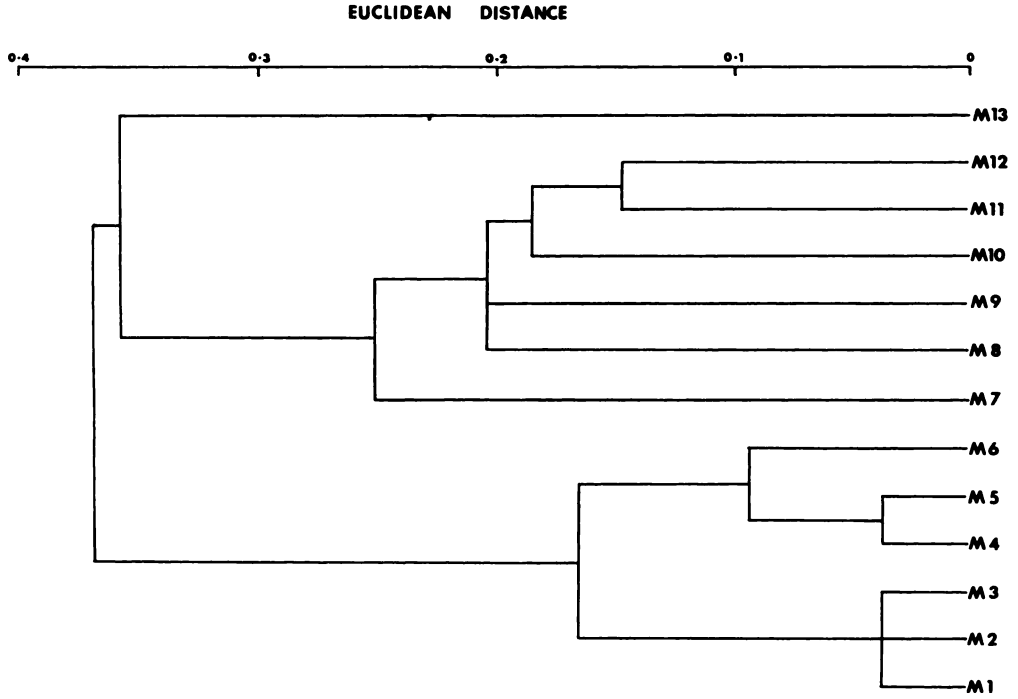

FIGURE 5. Phenogram of euclidean distances among zymodemes of *G. duodenalis*, clustered by group-average (UPGMA) strategy.

Identical patterns occurred in all the different subcultures of each isolate which were examined. Other isolates, prepared in exactly the same way, showed single-banded patterns for the same enzymes. It is therefore probable that the different isoenzymes in the multiple-banded patterns were genetically determined. In light of the results from previous studies,[18, 20, 21, 33] this requires some explanation.

The interpretation most consistent with these other studies is that isolates BAH12, BAH7, BAH16, and BAH20 contained genetically heterogeneous populations of haploid organisms. From the number of isoenzymes in the multiple-banded patterns (Fig. 4), this requires the presence of at least 3 genetically different haploid clones in isolates BAH12, BAH7, and BAH16 and 2 different clones in isolate BAH20. For all subcultures of each of these 4 isolates which were examined, the relative staining intensities of isoenzymes in the multiple-banded patterns were identical. If the isolates originally consisted of genetically different clones, this would require each subculture to contain similar quantities of each clone. Although intuitively this seems unlikely, it cannot be dismissed; firstly, because of possible imprecision in our visual measurements of staining intensities, and secondly, because we know little about the dynamics of growth of populations of *Giardia* in culture.

If the multiple-banded patterns of BAH12, BAH7, BAH16, and BAH20 do not result from the presence of genetically different haploid clones, a number of other interpretations should be considered. The isolates may be haploid, with isoenzymes specified by multiple loci, but this requires many mutations in duplicated genes.[32, 34] More information is needed on the structure and function of the two nuclei in *Giardia*. Alternatively, the isolates may be diploid (or polyploid), with isoenzymes specified by different alleles at the same locus. This seems possible given the recent evidence for diploidy and genetic exchange in *Trypanosoma*,[35, 36] *Plasmodium*,[37] and *Entamoeba*.[38] However, the present data are not adequate to confirm diploidy in *Giardia*. Except for PGM, multiple-banded enzymes did not always fit expected heterozygous patterns (although this also occurs in many organisms known to be diploid).[39] In addition, homozygous variants with bands corresponding to those of presumed heterozygotes were not found for any enzyme.

At this stage, a conclusive genetic interpretation of the banding patterns in our isolates of *Giardia* would be premature. To eliminate the possibility that isolates BAH12, BAH7, BAH16, and BAH20 contain genetically heterogeneous populations of haploid organisms, we need to clone individual trophozoites and examine their electrophoretic patterns. This is currently being attempted by initiating cultures from single trophozoites. However, from at least 20 attempts, no clones have been established in in vitro culture. It appears that *Giardia*, like other protozoa,[40] displays the "loneliness phenomenon" and may prove difficult to clone using a single organism to initiate cultures.

Although we cannot confidently assign genotypes to all electrophoretic variants, it is clear that considerable genetic heterogeneity exists among the isolates examined in the present study. Very few isoenzymes were shared by the 3 principal groups of zymodemes identified by cluster analysis. Groups I and III had no bands in common for any enzyme; groups I and II shared 6.2% of bands for 3 out of 10 enzymes; and groups II and III shared 10.3% of bands for 4 out of 10 enzymes. Without a conclusive genetic interpretation of banding patterns, these data cannot be related to measures of genetic distance between recognized taxa in other organisms,[41, 42] but they are of the order which has been used to confer specific status in *Trypanosoma*[43] and *Leishmania*.[44] We have decided to suspend any taxonomic decisions pending further genetic studies of our isolates of *Giardia*.

The present study confirms the suspicion from previous electrophoretic and restriction endonuclease analyses that the morphologically defined species, *G. duodenalis*, encompasses a wide spectrum of genetic heterogeneity. From our results, and those of other workers,[18–20] there appears to exist a group of organisms, genetically very similar to the Portland isolate (P1; ATCC 30888), distributed virtually worldwide. While some, usually local, variants differ from this group at only a few enzyme loci or restriction sites (e.g., zymodemes M1, M2, M3 of our study, zymodeme II of Bertram et al.,[18] isolates N and AB of Nash et al.[19]), others are genetically very different (e.g., zymodemes M7–M13 of our study, zymodeme III of Bertram et al.,[18] isolates Be-1, CM, and GS of Nash et al.[19]).

This genetic variation has important implications, not only for the taxonomy of *Giardia*,

but also for the epidemiology of giardiasis. Similar investigations in which isoenzyme analysis has been applied to the causative agents of trypanosomiasis and leishmaniasis have helped to correlate divergent epidemiological characteristics with genetically distinct populations of the parasites.[45] Results of the present study have demonstrated that genetically distinct "strains" of *G. duodenalis* are not restricted to a particular species of host and that cross-transmission is therefore possible. The isolates from cats (BAC1, BAC2, BAC3, P1) were genetically identical, or very similar to each other and many human isolates. Previous studies have found little difference between isolates from humans, cats, guinea pigs, rabbits, and beavers.[18, 19, 33] It therefore seems likely that zoonotic reservoirs of *Giardia* infection do exist. Although the rodent isolate (BAR1) was genetically distinct from all others, it fell within the range of variation found between different human isolates, e.g., between human isolates BAH3 (zymodeme M1) and BAH7 (zymodeme M7). In contrast to the majority of isolates from humans and cats, but in common with isolates from dogs,[11] BAR1 has repeatedly failed to establish in vitro.

The epidemiological significance of the variation between human isolates of *Giardia* remains to be determined. There is no obvious geographic correlation; isolates sharing the same zymodeme originated from widely separated localities, while isolates with very different zymodemes came from the same areas (Fig. 1). It has been suggested that intraspecific variation in *G. duodenalis* may be associated with differences in clinical aspects of giardiasis or the response of the parasite to chemotherapy.[12] Although distinct "strains" have been identified on the basis of antigenic, biochemical, or molecular variation, such differences have not yet been linked to clinical manifestations or virulence properties. It is unlikely that genetic variants of *Giardia* exhibit identical virulence characteristics and that the clinical course of giardiasis is solely determined by the individual host.

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